EFFECT OF SPAN 80 - TWEEN 80 MIXTURE COMPOSITIONS ON THE STABILITY OF SUFLOWER OIL-BASED EMULSIONS

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CERTIFICATE

This is to certify that this thesis entitled, "**EFFECT OF SPAN 80 - TWEEN 80 MIXTURE COMPOSITIONS ON THE STABILITY OF SUNFLOWER OIL-BASED EMULSIONS**" submitted by **Mr. ANKURMAN SHRESTHA** in partial fulfillment for the requirements for the award of Bachelor of Technology Degree in Biotechnology at National Institute of Technology, Rourkela is an authentic work carried out by him under my guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University / Institute for the award of any Degree or Diploma.

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CONTENTS

Abstract

The study describes the effect of span 80-tween 80 surfactant mixtures on the stability of the sunflower oil based emulsions. The emulsification was carried out in various proportions of span 80-tween 80 mixtures so as to have a HLB range varying from 6.4 to 13.8. Attempts were made to prepare both W/O and O/W emulsions by using oil: water fractions at 4:1 and 1:4 ratios, respectively. The emulsions which were stable for 24 h, were further observed for 56 h to have an insight on their long-term stability. The emulsions which showed signs of destabilization, during the period of 24 h to 56 h, were analyzed microscopically and measuring pH to understand the mechanism of destabilization. The stable samples were thoroughly characterized by light microscopy, pH measurement and accelerated stability test by thermocycling method. The micrographs of the samples were analyzed using Image J and Vision Assistant softwares. All samples stable up to 24 h were found to be W/O emulsions by dye solubility test. The unstable and short-term stable samples indicated cracking and creaming/sedimentation, respectively, as the destabilization phenomena. The pH of the short-term stable samples decreased with time whereas the pH of the long-term stable samples was almost constant throughout the 56 h time period. The long-term stable emulsions did not show any change in the particle size along the time scale.

Keywords: Span 80, Tween 80, Sunflower oil, Emulsion, Stability, Image J, Vision Assistant

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Chapter 1

INTRODUCTION

1.1 Introduction

Emulsions are defined as the biphasic systems consisting of two immiscible liquids where one of the liquid phases is dispersed in the other[1]. They can be categorized into two types, *viz.* oil-in-water (O/W) and water-in-oil (W/O) emulsions. O/W emulsion has water as the external phase while oil forms the internal phase whereas W/O emulsion consists of water droplets dispersed within oil continuous phase[2]. Emulsions have found numerous applications in the food, cosmetics and pharmaceutical industries. Emulsions, in general, have a cloudy appearance and are basically white in color due to the scattering of light passing through it. The emulsions are inherently thermodynamically unstable. The long term stability of emulsions is one of the major issues associated with its commercial applications[3]. To improve its stability, various emulsifiers (e.g. emulsifying wax, cetearyl alcohol, polysorbate 20 and ceteareth 20) are used. The emulsifiers help in reducing the interfacial tension amongst the two phases and help in making the system a thermodynamically stable one [4].

1.2 Objective of the report

The current study deals with the evaluation of the stability of the emulsions prepared by varying the composition of the emulsions. The sunflower and water based emulsions were prepared having sunflower oil: water proportions of 1:4 and 4:1. Span 80-tween 80 surfactant mixtures, both being elastic film-forming surfactants, of varying proportions were used as emulsifiers. In-depth analysis of the emulsion was done by using image processing software (Image J and Vision Assistant 2010) apart from the accelerated stability tests [5]. The pH of the stable and destabilized samples was compared with respect to time.

Chapter 2

MATERIALS & METHODS

2.1 Materials

Tween 80 (polyoxyethylene sorbitan monooleate) and rhodamine-B was procured from Himedia, Mumbai, India. Span 80 (Sorbitan monooleate) was obtained from Loba chemie, Mumbai, India. Sunflower oil (SO) was purchased from the local market. Double distilled water was used throughout the study.

2.2 Preparation of emulsions

The emulsifiers were prepared using the surfactants span 80 (HLB= 4.3) [6]and tween 80 (HLB 15.0). The emulsifiers were prepared by mixing of span 80 and tween 80 [7] in various weight proportions to have HLB values ranging from 6.4 to 13.8 [8]. The surfactants were homogenized for 20 min at 600 rpm using a magnetic stirrer. The compositions of the emulsifiers and their HLB value have been tabulated in table 1. The HLB values of the emulsifiers have been calculated as per the equation 1.

$$HLB_{\text{Resultant}} = \left[\frac{(HLB_1 \times W_1) + (HLB_2 \times W_2)}{100} \right] \quad (1)$$

where,

$HLB_{Resultant}$= HLB of the emulsifier obtained by mixing span 80 and tween 80,

HLB_1= HLB value of span 80,

HLB_2= HLB value of tween 80,

W_1= Weight% of span 80, and

W_2= Weight% of tween 80

Table 1: Resultant HLB values

Serial No.	Span 80: Tween 80 [w/w]	$HLB_{Resultant}$
1	4:1	6.4
2	2:1	7.8
3	4:3	8.9
4	2:3	10.7
5	1:2	11.5
6	1:4	12.9
7	1:8	13.8

The emulsions were prepared by varying the SO: water ratios so as to have 1:4 and 4:1 fractions. The emulsifiers were homogenized either with water or SO with continuous vortexing for 1 min. This was followed by the addition of the second phase and subsequent vortexing for another 1 min. The compositions of the emulsions have been tabulated in table 2. The prepared emulsions were observed at room-temperature for 24 h. If no phase separation was observed, then the emulsion was used for further analysis. As per the reported literature, the emulsions stable for the first 24 h is usually stable for several days[9]. The stable emulsions were kept at room-temperature for an extended period of 56 h to figure out any visual destabilization indicator. The samples which showed any signs of destabilization during this period were regarded as short-term stable samples while samples which did not destabilize in this period were regarded as long-term stable emulsions [10].

Table 2: Emulsion composition

Sample code	Oil/water (w%/w%) [g/g]	Surfactant HLB	Wt. surfactant (15% of 20% phase)	Surfactant premixed with	Phase added
E1	80 : 20 [8:2]	6.4	0.3	Water	Oil
E2	80 : 20 [8:2]	6.4	0.3	Oil	Water
E3	20 : 80 [2:8]	6.4	0.3	Water	Oil
E4	20 : 80 [2:8]	6.4	0.3	Oil	Water
E5	80 : 20 [8:2]	7.8	0.3	Water	Oil
E6	80 : 20 [8:2]	7.8	0.3	Oil	Water
E7	20 : 80 [2:8]	7.8	0.3	Water	Oil
E8	20 : 80 [2:8]	7.8	0.3	Oil	Water
E9	80 : 20 [8:2]	8.9	0.3	Water	Oil
E10	80 : 20 [8:2]	8.9	0.3	Oil	Water
E11	20 : 80 [2:8]	8.9	0.3	Water	Oil
E12	20 : 80 [2:8]	8.9	0.3	Oil	Water
E13	80 : 20 [8:2]	10.7	0.3	Water	Oil
E14	80 : 20 [8:2]	10.7	0.3	Oil	Water
E15	20 : 80 [2:8]	10.7	0.3	Water	Oil
E16	20 : 80 [2:8]	10.7	0.3	Oil	Water
E17	80 : 20 [8:2]	11.5	0.3	Water	Oil
E18	80 : 20 [8:2]	11.5	0.3	Oil	Water
E19	20 : 80 [2:8]	11.5	0.3	Water	Oil
E20	20 : 80 [2:8]	11.5	0.3	Oil	Water
E21	80 : 20 [8:2]	12.9	0.3	Water	Oil
E22	80 : 20 [8:2]	12.9	0.3	Oil	Water
E23	20 : 80 [2:8]	12.9	0.3	Water	Oil
E24	20 : 80 [2:8]	12.9	0.3	Oil	Water
E25	80 : 20 [8:2]	13.8	0.3	Water	Oil
E26	80 : 20 [8:2]	13.8	0.3	Oil	Water
E27	20 : 80 [2:8]	13.8	0.3	Water	Oil
E28	20 : 80 [2:8]	13.8	0.3	Oil	Water

2.3 pH variations of the emulsions

The variation in the pH of the stable emulsions was measured with the help of a digital pH meter (model 132E, EI products, India). The pH values were noted down at intervals of 8 h for 56 h.

2.4 Determination of emulsion type

Dye solubility test was performed on the long-term stable emulsions to find out the emulsion type. The dye solubility test involves the addition of a pinch of rhodamine-B dye on to the surface of the emulsions. If the emulsion is of W/O type, then the rhodamine-B dye will float on the emulsion surface, else the dye will dissolve thoroughly causing a change in the color of the emulsion from white to pink.

2.5 Accelerated thermal stability test

The accelerated thermal stability tests were carried out on the long-term stable emulsions by thermocycling method. The stability of the pharmaceutical products may be carried out by thermo-cycling process. The samples which were stable for the first 24 h, were used to conduct accelerated stability test. Alternate heating and cooling of emulsions for a particular period can help estimating the stability of the emulsions. The emulsions were alternatively incubated for 15 min at temperatures of 50^{o}C and -20^{o}C. The samples were regarded as stable samples, if the emulsions withstand at least 5 cycles of the thermo-cycling process. The study was extended for 16 cycles to have an idea on the long-term stability of the emulsion.

2.6 Microscopy

The microstructures of the emulsions were studied under compound light microscope (CH20i, Olympus, India). The microstructures of the emulsions were analyzed using ImageJ and NI Vision Assistant softwares. All the stable samples were examined under microscope for 56 h at an interval of 8 h.

Chapter 3

RESULTS AND DISCUSSIONS

3.1 Preparation of emulsions

The emulsions were prepared in 10 ml glass culture bottles (figure 1). The emulsions were then kept aside without disturbing, at room temperature, for 24 h. All the emulsion with SO: water ratio of 1:4 (E3, E4, E7, E8, E11, E12, E15, E16, E19, E20, E23, E24, E27 and E28) showed phase separation, immediately after vortexing, for all HLB values indicating that the emulsifiers did not promoted O/W emulsion formation. The emulsions E21 and E22, whose SO: water ration is 4:1, also destabilized immediately. The destabilization of the emulsions may be attributed to the cracking of the emulsions [11]. This may be due to the merging of smaller droplets to a bigger one, which results in the removal of the continuous phase from the space available within the two droplets and subsequent rupture of the emulsifier barrier (due to the hydrodynamic pressure exerted by the continuous phase) leading to the coalescence of the smaller droplets. This process continues till the oil and water phase separates to form distinct layers[12]. This kind of destabilization has been accounted to the inability of the emulsifiers to prevent the merger of the smaller droplets [13]. Twelve emulsions were found to be stable for 24 h. They were subsequently kept undisturbed at room-temperature up to 56 h. Six emulsions (E5, E9, E13, E17, E21 and E26) showed destabilization which may be attributed to the process of sedimentation or creaming (figure 2). This happens when dispersed phase forms floccules, which then either settle down or moves up due to the influence of the gravity on the phase with higher density. Since these emulsions are forming sediments, they are W/O emulsions[14]. Water being higher density phase settles down[15]. These short-term stable emulsions were discarded after preliminary examination to understand the mechanism of destabilization. The rest of the samples (E2, E6, E10, E14, E18 and E25) were found to be stable for even after 9 months of storage at room-temperature (figure 3).

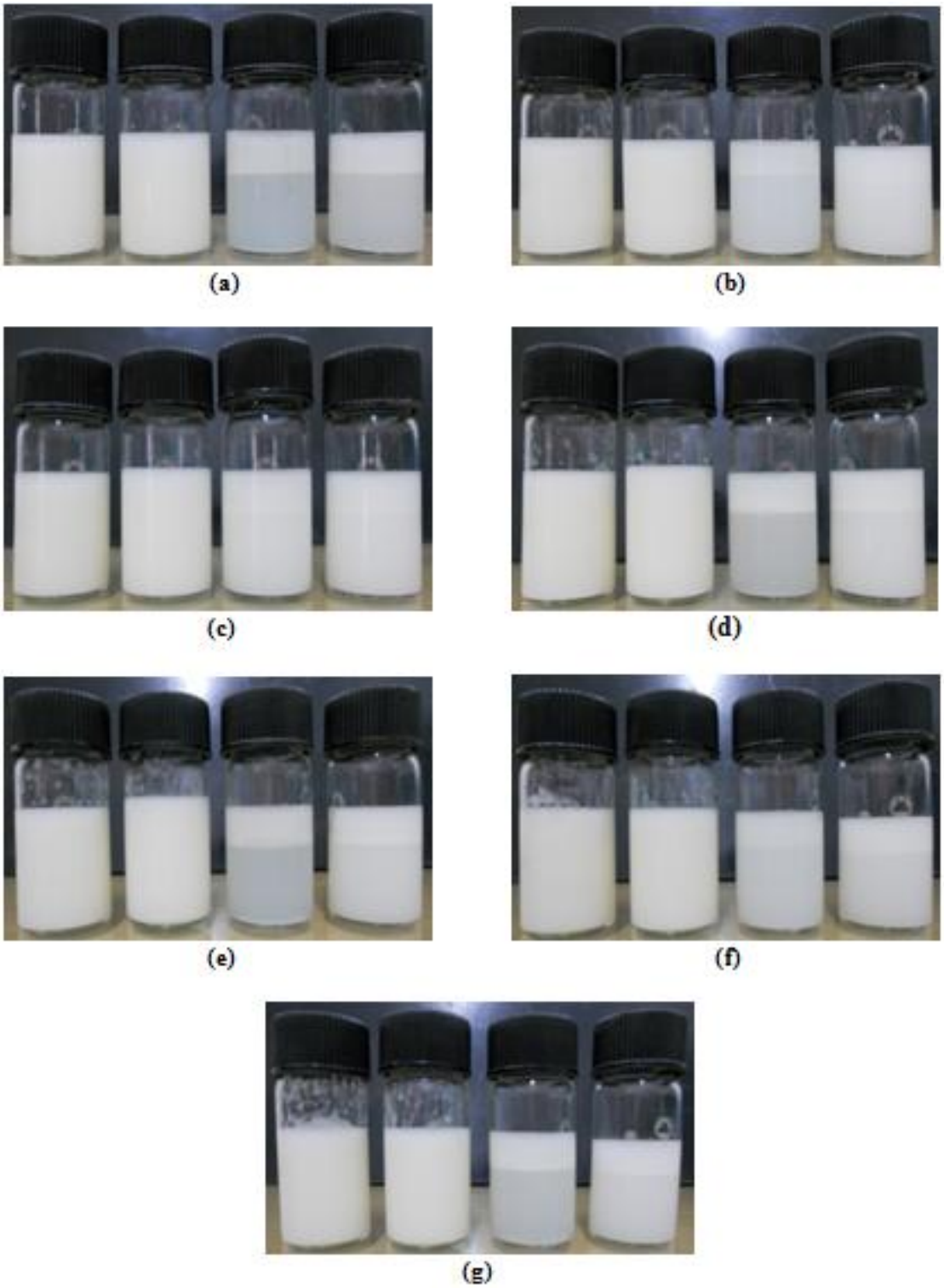


Figure 1. Sunflower oil & water based emulsions. (a) E1-E4 (HLB 6.4) (b) E5-E8 (HLB 7.8) (c) E9-E12 (HLB 8.9) (d) E13-E16 (HLB 10.7) (e) E17-E20 (HLB 11.5) (f) E21-E24 (HLB 12.9) (g) E25-E28 (HLB 13.8)

Figure 2: Short-term stable emulsions showing sedimentation. (a) E5 (b) E9 (c) E13 (d) E17 (e) E21 (f) E26

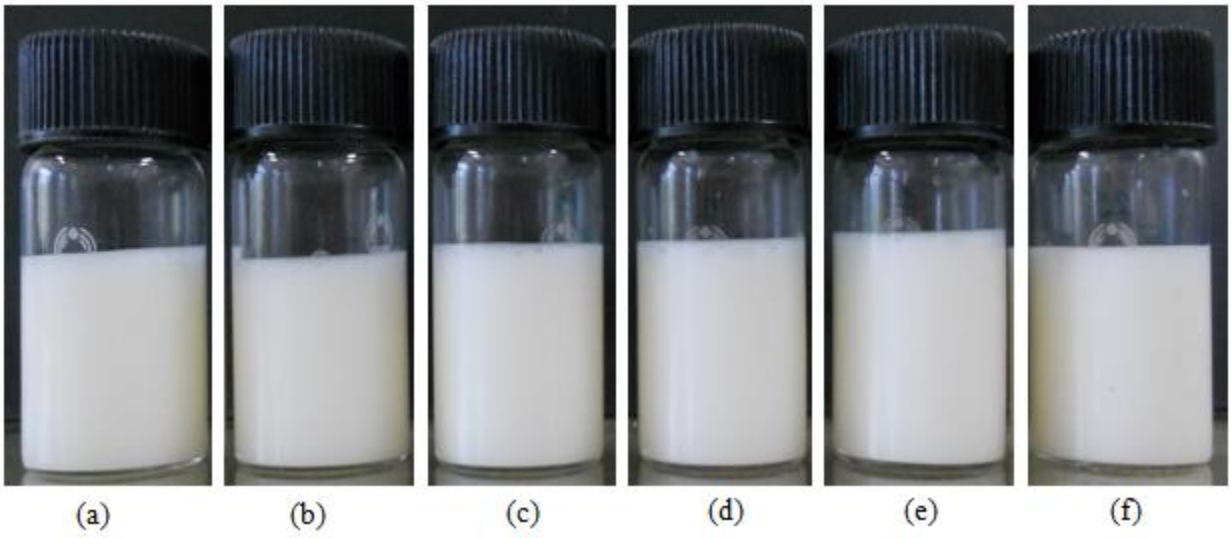
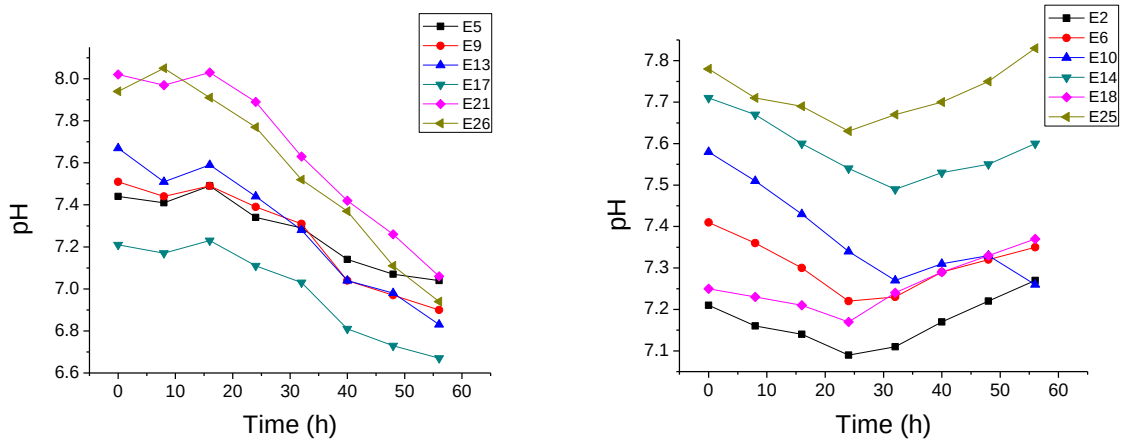


Figure 3: Long-term stable emulsion samples. (a) E2 (b) E6 (c) E10 (d) E14 (e) E18 (f) E25

3.2 pH measurement

The pH of the freshly prepared emulsions, which were found to be stable for the first 24 h, was measured for 56 h at an interval of 8 h (figure 4). The results indicate that the pH of the long-term stable emulsions (E2, E6, E10, E14, E18 and E25) were constant, within a certain limit, whereas the short-term stable emulsions (E5, E9, E13, E17, E21 and E26) indicated a continuous decrease in the pH values.



(a) **(b)**

Figure 4: pH variation of emulsions. (a) Short-term stable emulsions, and (b) Long-term stable emulsions.

3.3 Determination of emulsion type

To figure out the type of the emulsions, the long-term stable emulsions were subjected dye solubility tests [16]. The test suggested that the long-term stable emulsions were W/O type of emulsion, which showed that the rhodamine-B dye did not get dissolved in the external phase, but remained as powdered clusters on the surface of the emulsions (figure 5). The results present an interesting finding. In general, surfactants having HLB values of 0-8.6 promote W/O emulsion[17] whereas surfactant with HLB values > 9.6 promote O/W emulsions. In the present study, when the HLB value was modulated, in the range of 6.4 to 13.8, by mixing various proportions of span 80 and tween 80, the resultant emulsifier promoted W/O emulsion irrespective of the HLB value.

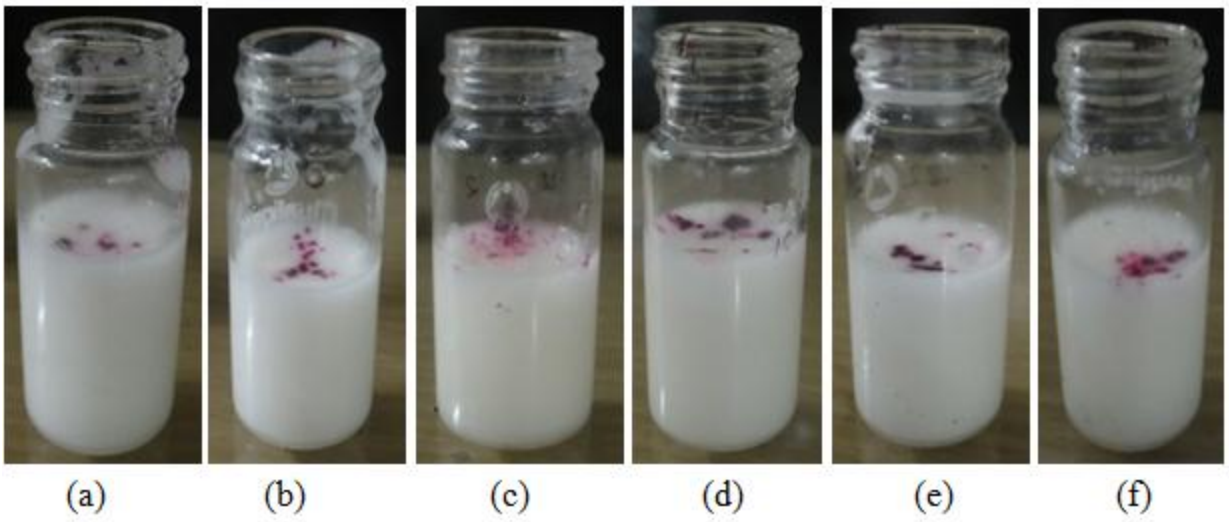


Figure 5: Dye solubility test for the long-term stable emulsions. (a) E2 (b) E6 (c) E10 (d) E14 (e) E18 (f) E21 (g) E25

3.4 Accelerated thermal stability testing

The accelerated thermal stability testing of emulsions were carried out on the long-term stable emulsions. Emulsions are often regarded as complex systems due to the presence of various factors which might affect the stability of the emulsion [18]. Due to this reason, the Arrhenius relationship cannot be used to predict stability of the emulsions though it has been successful in predicting the stability of a number of simple pharmaceutical, nutraceutical and cosmetics formulations. Instead of this, freeze-thaw method has become popular for predicting the stability of the emulsion formulations. The method employs continuous exposure of the samples to a freeze-thaw cycle at short intervals of time with the minimum temperature being $\leq$ -5 ^{o}C and the higher temperature being dependent on the type of formulation. In general, the higher temperature is set at $\geq$ 50 ^{o}C. The thermocycling method is only a predictive method and do not return with the exact time interval of destabilization. The testing considers the probable change in the properties of the surfactants at elevated temperatures, which in turn may alter the partitioning property of the surfactant, and the probable rupture of the surfactant layer in the presence of ice crystals at lower temperatures [19]. In either of the case, the samples will become unstable. In general, it is considered that the samples should withstand at least 5 cycles of freeze-thawing process. The long-term stable emulsions (E2, E6, E10, E14, E18 and E25) were able to

sustain much more than 5 cycles indicating the inherent stability of the long-term stable emulsions (table 3).

Table 3: Accelerated thermal stability test

Emulsion code	No. of stable cycles	Time of Destabilization (h)
E2	9	4.5
E6	8	4
E10	13	6.5
E14	10	5
E18	8	4
E25	9	4.5

3.5 Microscopic studies conducted on the emulsions

The long-term stable emulsions were observed under a compound optical microscope (figure 6). The micrographs of the emulsions indicated that most of the dispersed phase was present as circular droplets and had a range of sizes rather than droplets of a particular size. This generally occurs for the emulsions, which are prepared by physical methods like vortexing and mechanical homogenizers [20].

The sizes of the circular droplets were calculated using ImageJ and NI Vision Assistant softwares from the micrographs. Figure 7 identifies the different steps used for the determination of the droplet sizes. Figure 8 shows the frequency droplet distribution graph. The results support the visual examination of the micrographs indicating the presence of a broad range of droplet size distribution rather than a narrow size range. It showed that the droplet sizes varied from 0.1 to 1.5 µm with a greater number of droplets being clustered in the range of 0.2-0.5 µm.

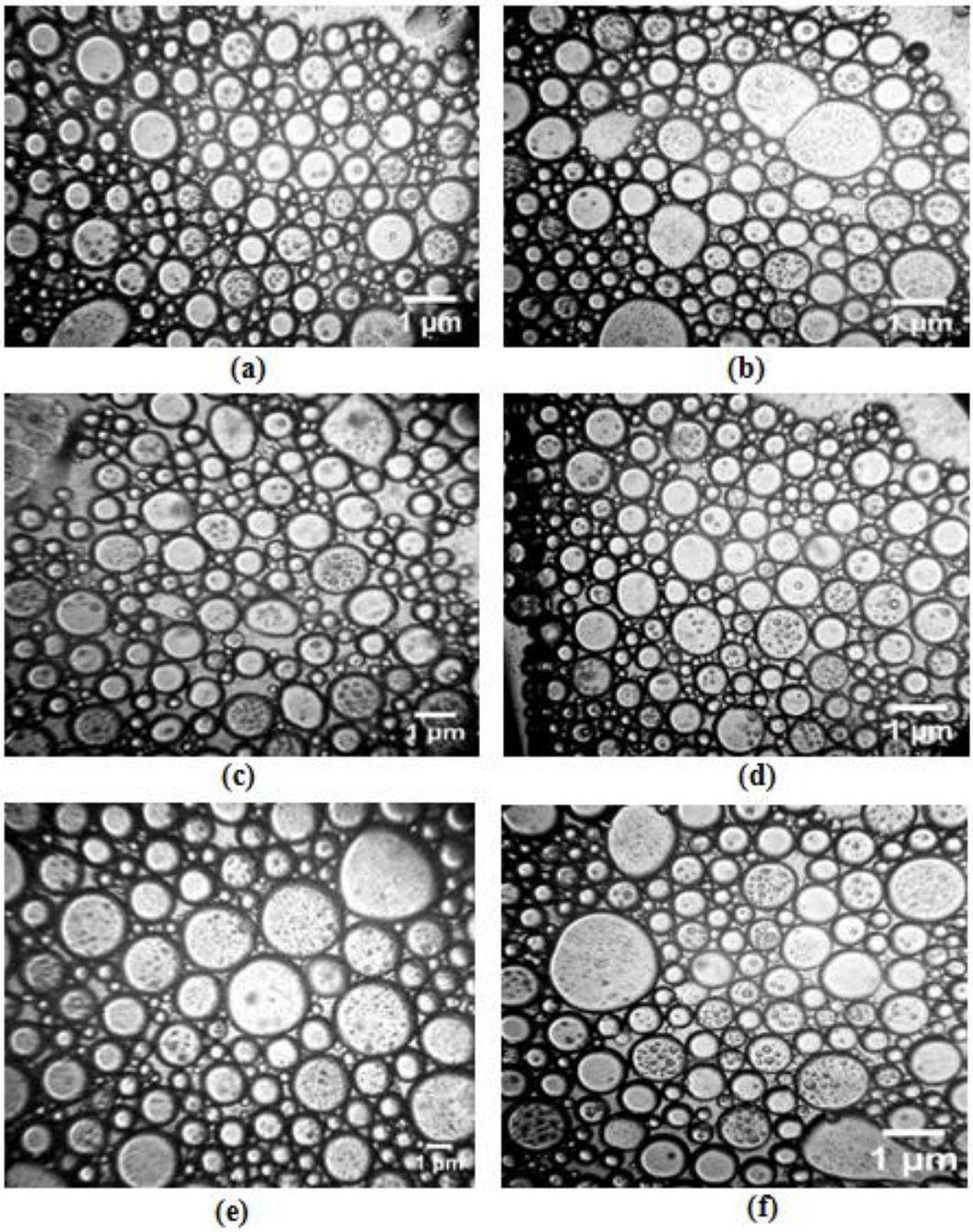


Figure 6: Light micrographs of stable emulsion samples. (a) E2 (b) E6 (c) E10 (d) E14 (e) E18 (f) E25

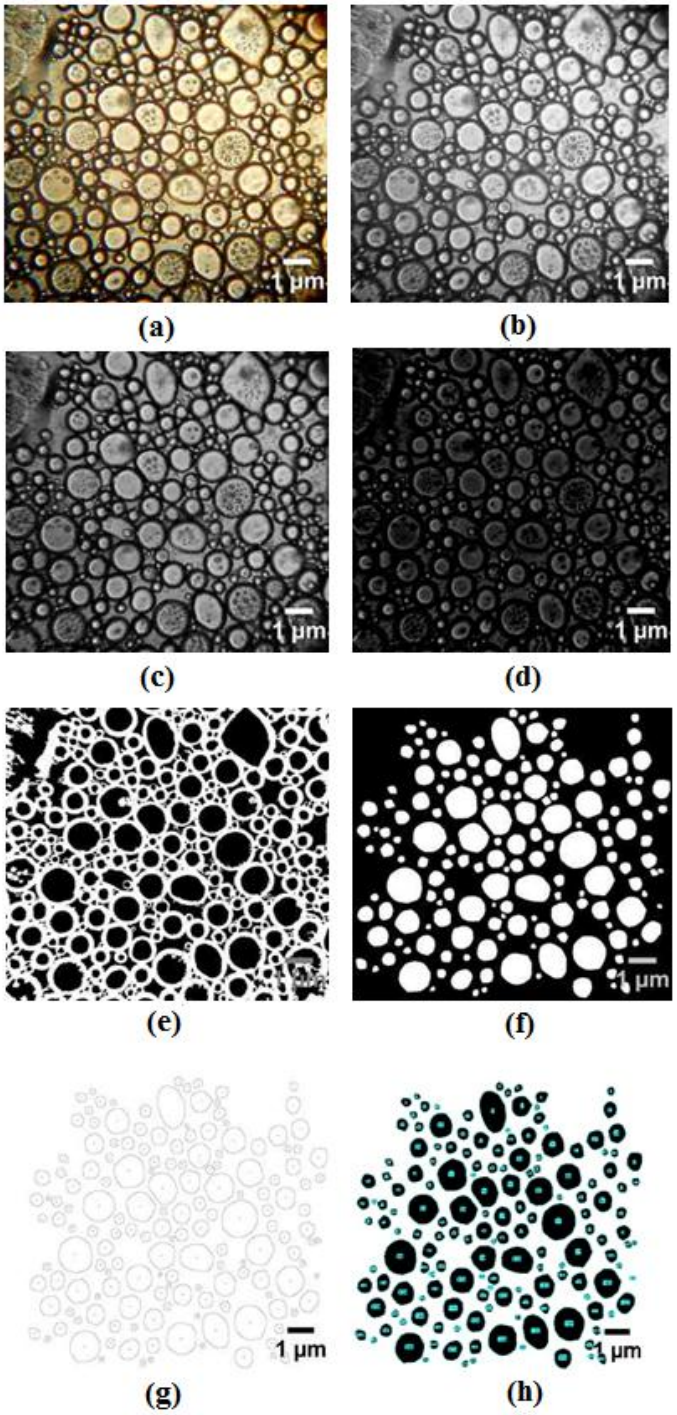


Figure 7: Steps involved in Image analysis using ImageJ and Vision Assistant. (a) Raw RGB microscopic image (b) Conversion of RGB image into 8-bit image (c) Background subtraction (d) Image calculator (e) Thresholded image (f) Vision assistant final image (g) Outline of analyzed particles (h) Analyzed Particles Count

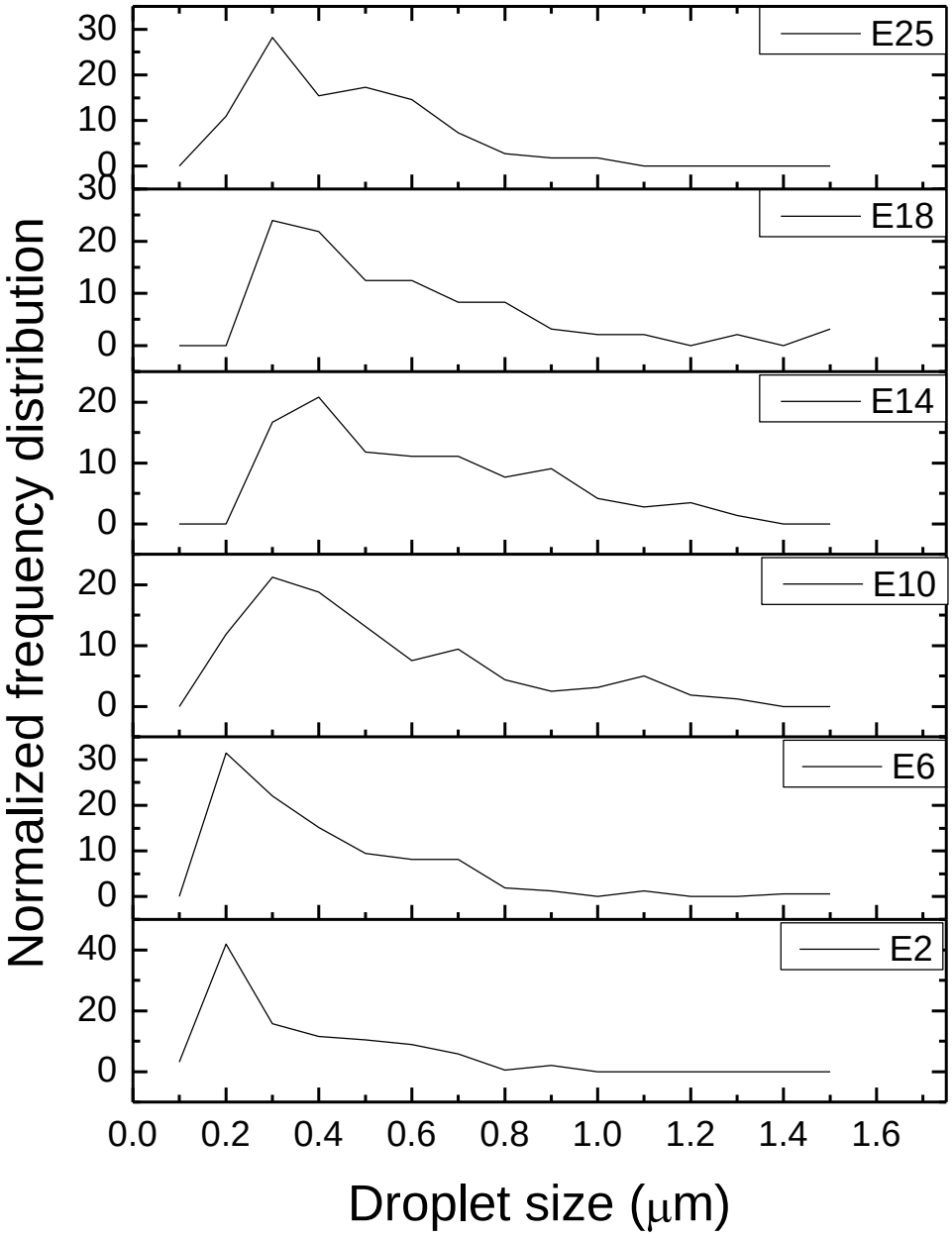


Figure 8: Normalized frequency distribution of the droplets

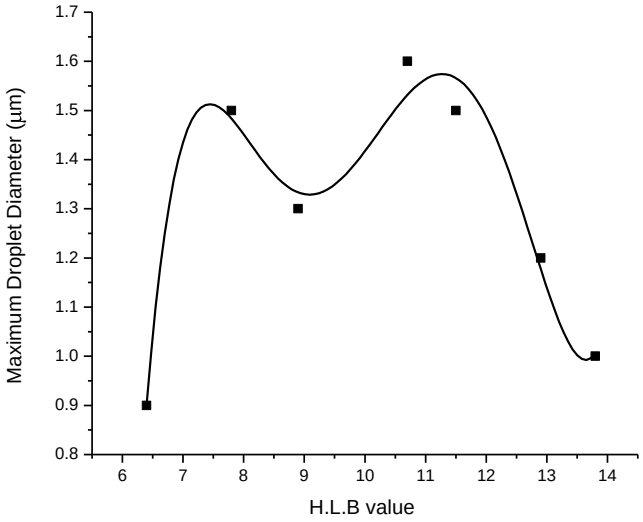


Figure 9: Variation of the highest proportion of droplet sizes as a function of HLB

Figure 9 shows the variation of the highest proportion of droplet sizes as a function of HLB value of the emulsifier [13]. The results indicate a bimodal distribution pattern. This suggests that as the HLB is increased from 6.4 to 7.8, there is an increase in the droplet size of the emulsions followed by a decrease in the droplet size as the HLB is increased to 8.9. With the subsequent increase in the HLB value to 11.5, there was an initial increase in the droplet size[21]. Thereafter, the droplet sizes of the emulsions decreased with the increase in the HLB value up to 13.8[22].

Figure 10 shows the micrographs of the E10 sample, taken as the representative sample for the long-term stable emulsions, sampled at an interval of 8 h for a period of 56 h. The samples were named as SX, X being the time of sampling in hours, e.g. S0 means sample withdrawn at 0 h and S56 means sample withdrawn at 56 h. Micrographs indicated that the droplets did not change its morphological shape and were circular in nature throughout the studies. The normalized frequency distribution pattern of the droplets indicated that there was insignificant change in the droplet sizes of the E10 emulsion even after 56 h (figure 11).

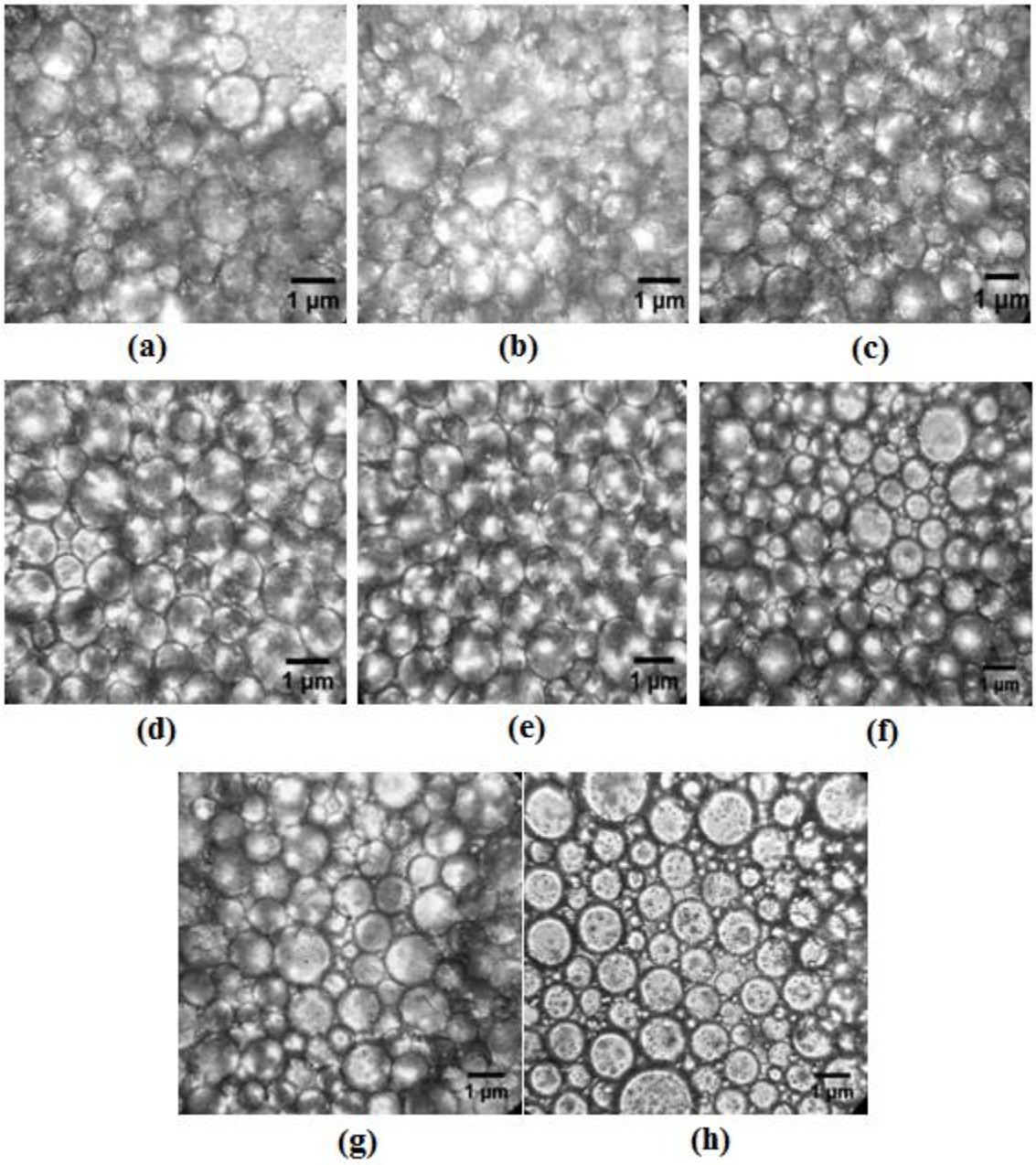


Figure 10: Light micrographs of E10 taken regular intervals of 8 h. (a) S0 (b) S8 (c) S16 (d) S24 (e) S32 (f) S40 (g) S48 (h) S56

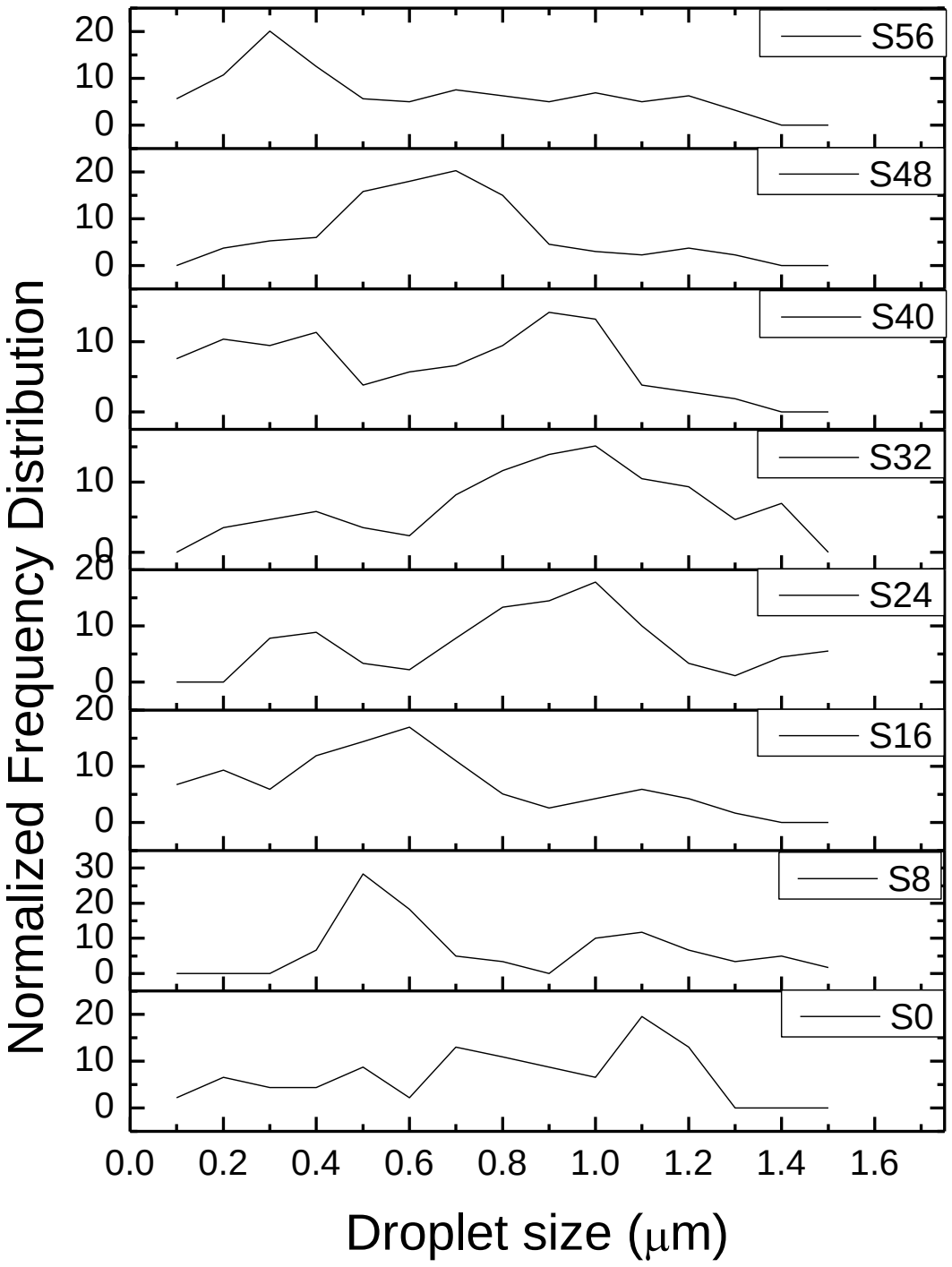


Figure 11: Normalized frequency distribution of the droplets of E10 on a time scale.

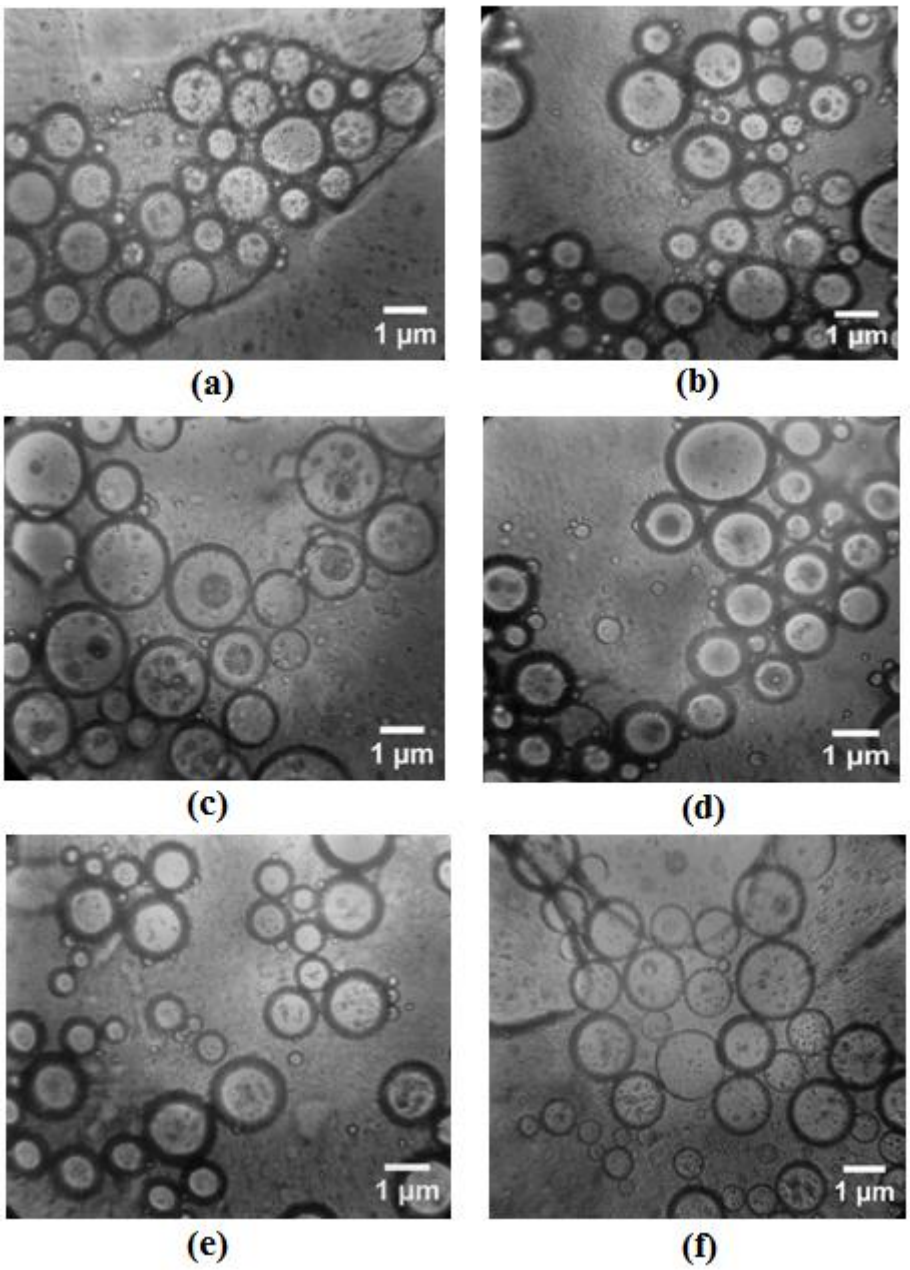


Figure 12: Micrographs of the short-term stable emulsions. (a) E5, (b) E9, (c) E13, (d) E17, (e) E21, and (f) E26

Figure 12 shows the microstructure of the short-term stable emulsions after 56 h reveals that the possible reason of destabilization is flocculation. The micrographs supports our previous assumption that the short-term stable emulsions were forming floccules, thereby behaving as single large entities. As these large entities are formed, they are being affected by the gravitational force and follows Stoke's law of sedimentation [15].

Chapter 4

CONCLUSION

4.1 Conclusion

The study reports the successful development of stable sunflower oil-water emulsions by simple vortexing method. The study indicated that as the HLB value was changed by mixing various proportions of span 80 and tween 80 in the range of 6.4 and 13.8, the emulsifier supported the formation of W/O emulsions even at higher HLB values. Cracking and sedimentation is the main reason of destabilization of the unstable and short-term stable emulsions, respectively. The long-term stable samples are stable even after 9 months of observation. The droplet size of the aqueous phase of the long-term stable emulsions was found to be in the range of 0.1-1.4 µm, thus making it suitable for controlled drug delivery [23].

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